

Lysolecithins as endothelium-dependent vascular smooth muscle relaxants that differ from endothelium-derived relaxing factor (nitric oxide)

(guanylate cyclase/phospholipids/lysophosphatidylcholine/endothelial cells)

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ABSTRACT The effects of lysolecithin (lysophosphatidylcholine) derived from egg yolk as well as of synthetic lysolecithins with different aliphatic chain lengths on tension development of rabbit aortic strips were investigated. Lysolecithins caused slowly progressing, dose-dependent relaxation that was inhibited by hemoglobin, methylene blue, and nordihydroguaiaretic acid. Indomethacin caused no inhibition of relaxation. The degree of relaxation was endothelium-dependent and appeared to be related to the activation of guanylate cyclase [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2]. Superoxide dismutase failed to influence relaxation. Lysolecithins with the longest aliphatic chain were the most potent relaxants of aortic strips. The experiments suggest a role of lysolecithins through their weak detergent action on membrane dynamics of endothelial cells, resulting in the production of cyclic GMP and the relaxation of arterial smooth muscle. Lysolecithins differ in several respects from endothelium-derived relaxing factor. Endothelium-derived relaxing factor is an unstable humoral substance released from endothelium and is identical to nitric oxide, itself a labile substance causing vascular relaxation and cyclic GMP accumulation. Lysolecithins may represent a different type of endothelium-dependent muscle relaxant.

Endothelial cells are involved in the regulation of regional blood flow through production of an endothelium-derived relaxing factor or factors (EDRF) (1). The endothelium is also responsible for bradykinin-induced relaxation in renal and pulmonary arteries (2). An EDRF that is distinguished from prostanooids has been identified as nitric oxide (NO) (3, 4). In bioassay preparation, EDRF had the same half-life, speed of relaxation, and degree of instability as NO. In addition, superoxide dismutase (SOD) reduced the inactivation of both EDRF and NO. Like EDRF, NO effects can be inhibited by hemoglobin (4). Evidence is now overwhelming that the endothelium produces a factor that relaxes arterial smooth muscle via activation of guanylate cyclase [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2] with NO as mediator (5).

Lysophospholipids (lysolecithins), are formed from the diacylphospholipids through the action of phospholipases. Small amounts of lysolecithins are present in most membranes (6). In a preliminary communication we reported that lysolecithin, prepared by the action of phospholipase A₂ (phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4) from egg yolk L- α -phosphatidylcholine, [lysolecithin (EY)] is a consistent relaxant of rabbit aortic strips in the presence of endothelium (7). Similar to lysolecithin (EY), phospholipase A₂ also produced slow dose-dependent relaxations, which were, however, independent of the presence of endothelium (8).

The present paper explores three related subjects: (i) relaxation of rabbit aortic strips by lysolecithin (EY) in the presence and absence of endothelium, (ii) the inhibitory effect of hemoglobin, methylene blue, nordihydroguaiaretic acid (NDGA) and indomethacin on relaxation by lysolecithin (EY), and (iii) comparisons of the degree and time of relaxation induced by synthetic lysolecithins on intact aortic strip and their inhibition by hemoglobin and methylene blue. Lysolecithins were found to be potent, slow-acting vascular smooth-muscle relaxants that activate guanylate cyclase. Individual differences exist between different lysolecithins, dependent on the aliphatic chain length; lysolecithin-induced relaxation is endothelium dependent.

MATERIALS AND METHODS

Male New Zealand White rabbits weighing 2.1–3.2 kg were anesthetized with sodium pentobarbital (30 mg/kg) and heparinized with 500 international units/kg i.v. After tracheotomy, the rabbits were ventilated with a respirator (Bird Mark 10; Space Technology, Palm Springs, CA). Median sternotomy was performed, and the thoracic portion of descending aorta was removed and immersed immediately in warm (37°C) Krebs–Henseleit solution (9). After removal of adjacent connective and adipose tissue, the aorta was cut into rings of ≈ 3 mm in width, and transverse strips were prepared. In the endothelium-denuded artery, referred to as “rubbed preparation,” endothelium was removed by the intimal surface being gently rubbed with moistened filter paper wrapped around a wooden stick. These strips were mounted in an organ chamber of 20-ml capacity with both ends fastened (9); the lower end was tied to the bottom of the chamber, and the upper end was attached to an isometric strain transducer (UL-20-Gr, Shinkoh, Minebea, Tokyo). The chambers were oxygenated with 95% O₂/5% CO₂ by slow bubbling to prevent foaming. Strips were allowed to equilibrate for 60 min, and basal (resting) tension was adjusted to 1.5 g (8). Composition of Krebs–Henseleit solution was 143 mM Na⁺/5.94 mM K⁺/2.54 mM Ca²⁺/1.19 mM Mg²⁺/1.19 mM H₂PO₄⁻/127.84 mM Cl⁻/25 mM HCO₃⁻/1.19 mM SO₄²⁻/10 mM glucose.

Tension development was induced by addition of 10⁻⁵ M histamine to the organ chamber. After a steady state was reached, 10⁻⁶ M acetylcholine was added to induce endothelium-dependent relaxation, thus confirming the integrity of endothelium.

Lysolecithins used in this series were either synthetic, (palmitoyl, stearoyl, oleoyl, myristoyl, decanoyl, caproyl

Abbreviations: NDGA, nordihydroguaiaretic acid; EDRF, endothelium-derived relaxing factor; SOD, superoxide dismutase; lysolecithin (EY), egg yolk-derived lysolecithin.

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lysolecithins) or derived from egg yolk. The latter contained palmitoyl and stearoyl lysolecithins. The effect of lysolecithin (EY) on relaxation of unrubbed intact aortic strips was studied in 16 rabbits. After precontraction with 10^{-5} M histamine and administration of 10^{-6} M acetylcholine to the bath, the preparation was washed and 10^{-5} M histamine was again applied. After steady state had been reached, cumulative doses of lysolecithin (EY)— 10^{-7} M, 10^{-6} M, and 10^{-5} M—were added. The dose-response relationship of lysolecithin was then plotted, and the time course and the slope of relaxation was compared with that of acetylcholine. Experiments on rubbed strips were done similarly.

Hemoglobin or methylene blue (both at concentrations of 10^{-5} M), NDGA (3×10^{-5} M), and indomethacin (10^{-5} M) were added before precontraction with histamine. In six experiments the inhibitory effects of hemoglobin and methylene blue were observed after relaxation with lysolecithin when a steady state was reached. Oxyhemoglobin was prepared, according to Martin, by adding a 10-fold molar excess of reducing agent sodium dithionite to a 1 mM solution of commercial bovine hemoglobin in distilled water. Sodium dithionite was removed by dialysis against 100 vol of distilled water for 2 hr at 4°C (10).

Each synthetic lysolecithin was dissolved in 100 μ l of dimethyl sulfoxide (Me_2SO) on a watch glass and diluted in 0.9 ml of Krebs-Henseleit solution. The mixture was then stirred continuously with a glass rod until all particles had disappeared. Final concentration of Me_2SO in the organ chamber was 0.01%; at this concentration the effect of Me_2SO on strip contractility was reported to be absent (11). Data were expressed as mean \pm SEM; percent relaxation was determined by decrease in tension from histamine-induced contraction. The Student's *t* test (paired and unpaired as indicated) was used for comparing the degree of relaxation. All compounds were obtained from Sigma.

RESULTS

The Effect of Lysolecithin (EY) on the Relaxation of Unrubbed and Rubbed Aortic Strips. Fig. 1 illustrates that lysolecithin (EY) significantly relaxed the unrubbed aortic strip. The relaxation was dose dependent, ranging from 7.5% with 10^{-7} M to 61% with 10^{-5} M lysolecithin (EY) (Fig. 2). The change in tension with the highest concentration of lysolecithin (EY) (10^{-5} M) was slightly less than that produced by 10^{-6} M of acetylcholine (61% versus 68%) (Fig. 2). Each individual decline in tension was significant as compared with the preceding concentrations ($P < 0.001$); the greatest decline occurred between concentrations of 10^{-6} to 10^{-5} M. Figs. 1 and 2 illustrate that relaxation in rubbed arteries was diminished; this difference was significant at higher concentrations of lysolecithin (EY) (10^{-5} M).

The Effect of Hemoglobin, Methylene Blue, NDGA, and Indomethacin on the Relaxation of Lysolecithin (EY). In the unrubbed preparations, preaddition of hemoglobin before precontraction, significantly reduced the percent relaxation by lysolecithin (EY), (from 61.3% to 15.2%, $P < 0.001$) (data not shown). Methylene blue also inhibited relaxation from 61.3% to 4.6% ($P < 0.001$) (data not shown). Inhibition by NDGA of endothelium-dependent relaxation by a variety of relaxants were demonstrated by Chand and Altura (12). Inhibition by NDGA was significant (from 61.3% to 37.1%), but the difference was less ($P < 0.05$) (data not shown). Indomethacin did not prevent relaxation (data not shown). In the rubbed preparation no significant inhibition was noticed. Furchgott *et al.* reported that hemoglobin administration after relaxation by acetylcholine markedly increases tension (13). We also observed, that when hemoglobin or methylene blue were added to the preparation during a steady state after

relaxation with lysolecithin (EY), a marked increase in tension was seen (Fig. 1).

The Effect of Synthetic Lysolecithins. Fig. 3 compares the relaxing effects of synthetic stearoyl, palmitoyl, oleoyl, myristoyl, decanoyl, and caproyl lysolecithins (10^{-5} M) in the unrubbed preparation. Marked differences in the effect of various synthetic lysolecithins were noticeable. Stearoyl, palmitoyl, and oleoyl lysolecithins were the most active relaxants. The decline in tension with stearoyl, palmitoyl and oleoyl lysolecithin was 47, 37, and 36%, respectively, in opposing the effect of histamine. No statistical differences in the decline in tension was present between these compounds. Lysolecithins with smaller carbon numbers influenced tension to a lesser degree (myristoyl 25%, caproyl 21%, and decanoyl 16%) (Fig. 3). The difference between the relaxation induced by stearoyl, on the one hand, and myristoyl, decanoyl and caproyl, on the other hand, was significant ($P < 0.001$).

Pretreatment with hemoglobin and methylene blue significantly inhibited relaxation caused by synthetic stearoyl lysolecithin (Fig. 1).

DISCUSSION

The results illustrate that synthetic as well as lysolecithins (EY) are potent relaxants of vascular smooth muscle (Figs. 1–3); the degree of relaxation depends on the presence of endothelium (Figs. 1 and 2). Hemoglobin, methylene blue, and NDGA inhibit relaxation, whereas indomethacin is ineffective. It has been shown that methylene blue inhibits cGMP (5, 14, 15) and that hemoglobin blocks the relaxation and the increase of cGMP induced by acetylcholine (10). When methylene blue or hemoglobin are added after relaxation with lysolecithins, marked contractions are recorded (Fig. 1).

For these reasons it is likely that lysolecithins relax vascular smooth muscle by promoting cGMP accumulation. NDGA inhibition of the relaxing effect of a variety of relaxants has been shown by Chand and Altura (12). Inhibition of relaxation by NDGA is less than that resulting from methylene blue or hemoglobin, though still highly significant. NDGA is an antioxidant and an inhibitor of lipoxygenases and is an effective inhibitor of acetylcholine-induced relaxation in rabbit aortas. The inhibition by NDGA of lysolecithin-induced relaxation suggests some participation of lipoxygenases in the inhibitory process. Failure of indomethacin to inhibit relaxation indicates that the cyclooxygenase pathway is not involved in the relaxation process (16, 17). Inhibition of relaxation by hemoglobin and methylene blue demonstrates that cGMP is the second messenger in the relaxation of lysolecithin; a similar function of cGMP in the relaxation by EDRF and nitrovasodilators has been repeatedly described (15). Previous data published from this laboratory have furnished direct evidence of activation of guanylate cyclase by phospholipase A_2 , the enzyme responsible for the formation of lysolecithins from lecithin (8).

Relaxation induced by lysolecithin (EY) in the presence of endothelium appears to be connected to activation of guanylate cyclase. Gruetter and coworkers (18) have shown that removal of endothelium results in a 4-fold decrease on vascular endothelium is well known and is dependent on the activation of guanylate cyclase by EDRF or NO (19, 20). The link between muscarinic receptors and guanylate cyclase has been clearly demonstrated, although vascular endothelium is not obligatory for stimulation of cGMP formation (17). Most probably arterial endothelial cells generate a factor that activates guanylate cyclase.

Several significant similarities and differences exist between EDRF (NO) and lysolecithin-induced relaxation. One of the most conspicuous is the speed of relaxation compared

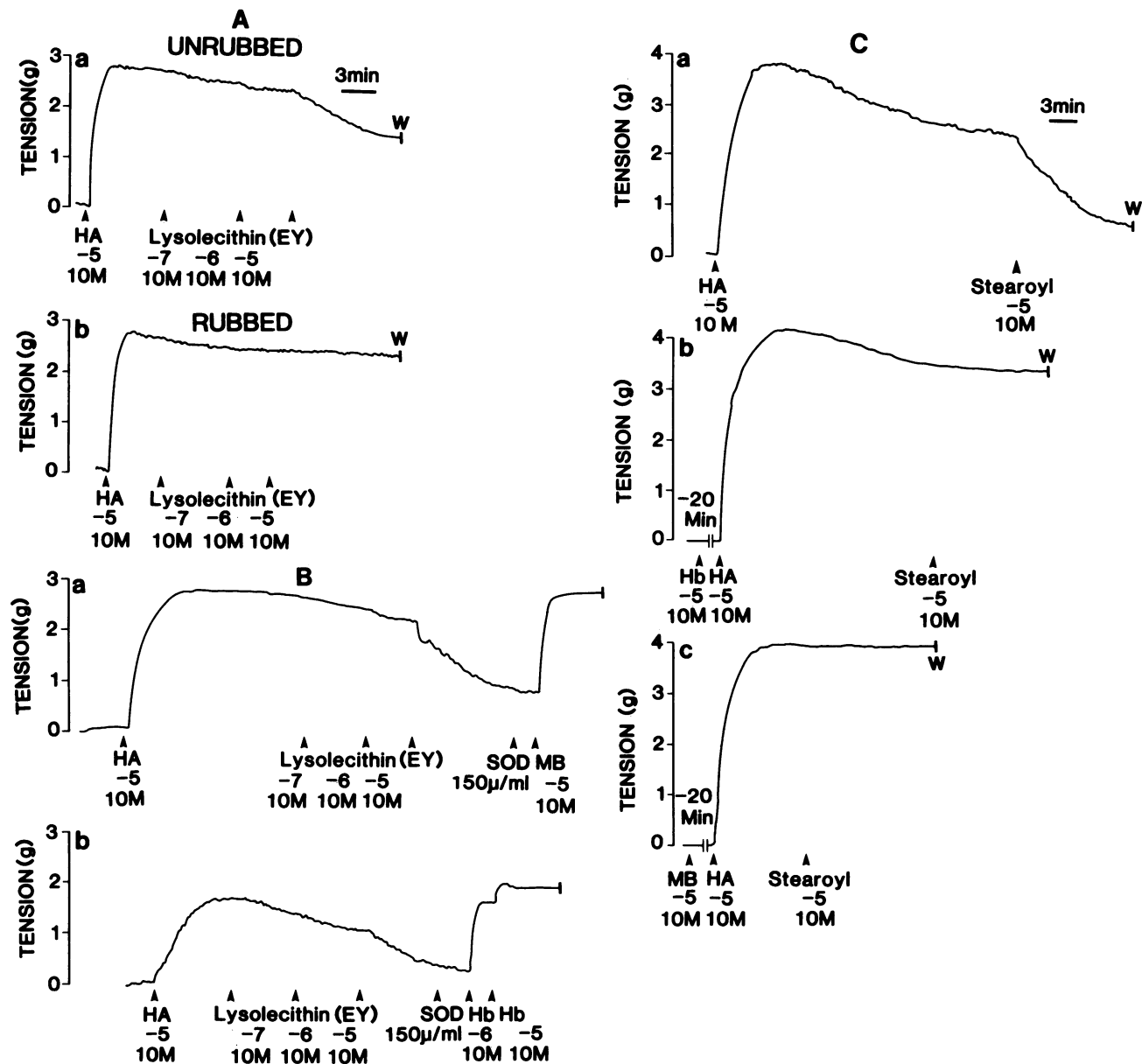


FIG. 1. Representative tracings of contractile changes of aortic strips after preconstriction with histamine (HA). (A) Difference in the effect of lysolecithin (EY) between rubbed and unrubbed aortic strips. No relaxation was noted in the rubbed preparation. (B) Dose-dependent relaxation by lysolecithin (EY) in unrubbed aortic strip. SOD was without effect in either upper or lower traces. The addition of methylene blue (MB) resulted in a marked increase in tension (upper trace). Similarly, hemoglobin (Hb) augmented tension particularly at higher concentrations (10^{-5} M, lower trace). (C) Synthetic lysolecithin (stearoyl, 10^{-5} M) caused marked relaxation (top trace). Addition of Hb and MB, added before preconstriction with histamine, inhibited relaxation (middle and bottom traces).

with acetylcholine (ACh). In order of magnitude the percent relaxation during the first 2 min is: ACh 63.2 ± 4.8 ; lysolecithin (EY) 23.8 ± 4.1 ; palmitoyl 13.7 ± 2.5 ; stearoyl 11.8 ± 2.7 ; oleoyl 12.2 ± 2.3 ; myristoyl 9.9 ± 2.4 ; decanoyl 4.6 ± 1.1 ; and caproyl 4.1 ± 1.1 .

A further important difference is the failure of SOD to potentiate relaxation (Fig. 1). In a previous publication we reported a small potentiating effect of SOD on lysolecithin (EY)-produced relaxation (7). However in the present experiments, the effect of SOD is either absent or negligible.

The common features between EDRF and NO and lysolecithins are inhibition by hemoglobin, methylene blue, and NDGA. Like EDRF or NO, indomethacin fails to inhibit relaxation. It is unlikely that prostacyclins are responsible for the relaxation induced by lysolecithins, because indomethacin fails to inhibit relaxation (21, 22).

Several previous publications have suggested that cleavage

of phospholipids and release of fatty acids are related to smooth muscle relaxation. Melittin, a potent activator of phospholipase A_2 and thimerosal, which inhibits lysolecithin acyltransferase, induce endothelium-dependent relaxations of rabbit aortas (22, 23). Like relaxations induced by lysolecithin, those resulting from thimerosal also developed more slowly than those induced by acetylcholine (23).

In line with the concept of the phospholipid origin of vascular smooth muscle relaxants are the results of Huang and Lee (24) and of Bing and Saeed (7, 8). Huang and Lee found that phospholipase A_2 relaxes aortic rings (24). In addition, relaxation by phospholipase A_2 was inhibited by methylene blue, suggesting a role of cGMP. Bing and Saeed found that phospholipase A_2 produced slow dose-dependent relaxation in both rubbed and unrubbed precontracted aortic strips, which is inhibited by hemoglobin and methylene blue (8). Although in some instances slight potentiation by super-

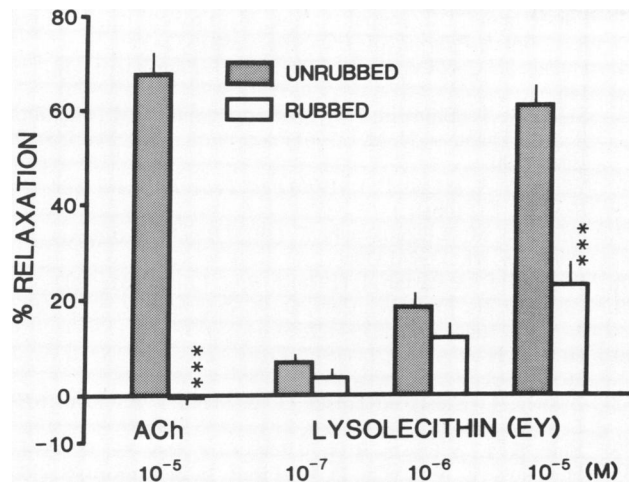


FIG. 2. Comparison of percent relaxation in intact (unrubbed) and endothelium-deprived (rubbed) aortic strips. The difference in the response to acetylcholine (ACh) was most marked ($P < 0.001$). At highest (10^{-5} M) concentrations of lysolecithin (EY) ($P < 0.001$) the difference between the two preparations was also highly significant.

oxide dismutase is noticeable, this effect is inconsistent and possibly represents an artifact.

Lysolecithins play an important role in the function of cell membranes. Normal components of biological membranes (1.8% of total membrane lipid in liver plasma membranes), they are present in tissue at concentrations of ≈ 1.5 mg per ml (25). Lysolecithins function as membrane transducers by diffusing rapidly through the lipid portion of the membranes to modify the activity of membrane-associated enzymes and alter the general properties of the membrane, such as fluidity and permeability (25). Lysolecithins are known to modify the activity of nucleotide cyclases. For example, several workers have recorded that soluble and insoluble guanylate cyclase activities are stimulated by lysolecithin and phospholipase A_2 (26). In addition, guanylate cyclase is stimulated by Triton X-100 in homogenates of rat small intestine (25). Some relationship may exist between the relaxation by surfactants such as Triton X-100 and lysolecithins (26). We have recently reported relaxation of aortic strips with this ionic detergent

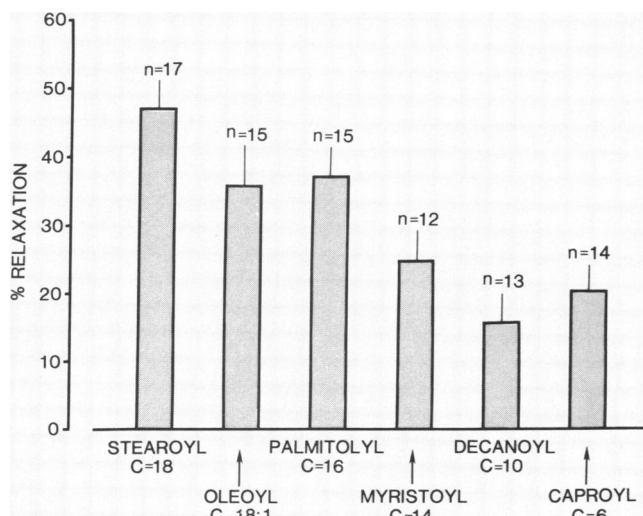


FIG. 3. Synthetic lysolecithins (stearoyl, oleoyl, palmitoyl, myristoyl, decanoyl, or caproyl lysolecithin at 10^{-5} M) relaxed un-rubbed aortic strips. The degree of relaxation depended on the aliphatic chain length. Lysolecithins with the longest aliphatic chain showed the greatest relaxation.

(7); Fujimoto and Okabayashi found that Triton X-100, similar to phospholipase A_2 , acts primarily on particulate guanylate cyclase and that perturbation of membrane architecture, rather than solubilization, accounts for stimulation of guanylate cyclase (27). Stimulation of guanylate cyclase activity by fatty acid and lysolecithin (28–31) was also described. These findings suggest a role of lysophosphatidylcholine in the regulation of the level of intracellular cGMP. Fink and Gross demonstrated that amphiphiles including palmitoyl lysophosphatidylcholine or L-palmitoyl carnitin, were quantitatively incorporated into sarcolemma, resulting in alterations of membrane molecular dynamics (32). These workers, using electron-spin resonance spectroscopy demonstrated that the sarcolemma contained a 4-fold higher grain density of incorporated lysophosphatidylcholine than either the mitochondrial or sarcoplasmic reticulum. The observed alterations in membrane fluidity were not produced by metabolites of amphiphilic compounds such as fatty acids or glycerophosphorylcholine.

The relationship of the amplitude of relaxation to the aliphatic chain length deserves some comments (Fig. 3). Accordingly, lysolecithins with the longest chain length are the most potent relaxants of the aortic preparation. Helenius and coworkers (33) have related the critical micellar concentration of synthetic lysolecithins to their aliphatic chain length. Apparently the lysolecithins with shorter aliphatic chains have higher critical micellar concentrations than those with longer aliphatic chains. Because the critical micellar concentrations represent the highest detergent chemical potential attainable, it follows that those lysolecithins with the lowest carbon number possess the highest chemical detergent potential. Therefore, the weaker the detergent, the greater its ability to relax aortic preparations. It must be remembered, however, that the action of detergents on membranes is complex, depending not only on detergent chemical potential, but also on the nature of the detergent, the kinetics of membrane-bound proteins, and changes in ion flux caused by perturbation of the lipid milieu of the membrane (32, 34).

In conclusion, it was found that lysolecithins are slow-acting relaxants of rabbit aortas. They activate guanylate cyclase, and relaxation is endothelium-dependent. Those lysolecithins with long aliphatic chain length possess the greatest relaxing activity, possibly related to the low degree of detergent activity. Therefore, lysolecithins differ in many respects from EDRF (NO) and may represent a different type of endothelium-dependent muscle relaxant.

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